Involvement of Neutrophil Phospholipase D in the Production of Reactive Oxygen Metabolites in Experimental Rat Colitis

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Background & Aims : Phospholipase D (PLD) hydrolyzes phosphatidylcholine and produces lipid second messengers including phosphatidic acid (PA) and diacylglycerol (DG). Although cellular PLD has recently been recognized as an important signal-transmitting enzyme, the roles of PLD in pathophysiological conditions are largely unknown. The aim of this study is to elucidate the roles of PLD in the production of reactive oxygen metabolites in experimental rat colitis.

Methods : Colitis was induced in rats by oral administration of dextran sulfate sodium. Luminol-amplified chemiluminences of the colonic mucosa and polymorphonuclear leukocytes were both measured.

Results and Conclusions : Oleate-dependent PLD was activated in the colitic mucosa. Chemiluminescence and myeloperoxidase activity of the colitic mucosa were significantly higher than those of the control normal mucosa. Preincubation of the mucosa *in vitro* with PLD, PA, or DG significantly enhanced chemiluminescence of the colitic, but not of normal mucosa. On the other hand, administration of glycerol, which inhibits the production of PA and DG by PLD, significantly decreased chemiluminescence induced by exogenous PLD. In rat polymorphonuclear leukocytes suspension, the addition of PLD, PA, or DG significantly increased chemiluminescence and propranolol significantly decreased the chemiluminescence induced by both PA and DG. These results suggest that PLD in neutrophils may be relevant to the production of reactive oxygen metabolites in experimental rat colitis.
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Key Words : Phospholipase D, Chemiluminescence, Colitis.

Introduction

Phospholipase D (PLD) hydrolyzes phosphatidylcholine (PC) to produce phosphatidic acid (PA) and choline. PA itself and diacylglycerol (DG), which is formed from PA by the action of PA phosphohydrolase, are recognized as important lipid second messengers in cells. It has recently been demonstrated that a number of hormones, growth factors, and other extracellular signals regulate PLD activity in intact cells.¹ Cellular PLDs have thus emerged as central components of signal transduction. However, their roles in pathophysiological conditions, especially in colonic inflammation, is largely unknown.² On one

hand, reactive oxygen metabolites (ROMs) are involved in inflammatory disease and are postulated to contribute to tissue injury in colitis.^{3~5} Stimulated polymorphonuclear leukocytes (PMNs) generate a number of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, hypochlorous acid, hydroxyl radicals and singlet oxygen.^{6,7} Two special enzymes, NADPH-oxidase and myeloperoxidase, are responsible for ROMs generation in neutrophils. Although it has recently been demonstrated that PLD is activated and its activation correlates with mucosal damage in experimental colitis in rats,⁸ further investigation is necessary to elucidate the mechanisms of action of PLD in colitis. This study examined the

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roles of PLD in the production of ROMs by measuring chemiluminescence in experimental rat colitis.

Materials and Methods

Dextran sulfate sodium (DSS, molecular weight 50,000), luminol, propranolol, glycerol, and casein were purchased from Wako Pure Chemical (Tokyo, Japan). 1,2-dioctanoyl-*sn*-glycerol (DG), 1,2-dioctanoyl-*sn*-glycero-3-phosphate (PA), 1-palmitoyl-2-[6-N-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl) amino] caproylphosphatidylcholine (C₆-NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, USA).

Induction of colitis.

Male Wistar rats (200–250 g) were obtained from Imai Experimental Animals (Saitama, Japan). Animals were kept in the Animal Resources Center of Gunma University. Unless otherwise stated, the rats were given free access to water and standard laboratory chow throughout the experimental period.

DSS was dissolved in tap water at a concentration of 5 % (w/v). This solution was given to rats as drinking water *ad libitum* for 7 days. Control rats were given tap water instead of DSS solution for the same period.^{9,10} The rats were sacrificed on day 7 of the treatment. An 8-cm segment of the distal colon was isolated, resected, and opened by a longitudinal incision on ice in preparation for the chemiluminescence and myeloperoxidase (MPO) assay.

Mucosal MPO activity.

After dissection, the mucosal surface of the colon was rinsed with ice-cold phosphate-buffered saline (PBS) and mucosal scrapings were homogenized with Diax 600 homogenizer (Heidolph, Germany) in 1.0 ml of buffer containing 0.5% hexadecyltrimethylammonium bromide, 50 mM potassium phosphate, pH 6.0. The homogenates were then sonicated for 10 seconds, freeze-thawed three times, and centrifuged at 40,000×g for 15 min at 4°C. An aliquot of the supernatant was taken for the determination of the enzyme activity according to Bradley et al.¹¹ Results were expressed as units per gram mucosal tissue.

Chemiluminescence of colonic mucosa.

Luminol-amplified chemiluminescence of colonic mucosa was measured using a Luminescence Reader (Model BLP 102; Aloka Inc., Tokyo, Japan) by the method previously described.⁴ Excised specimens from the inflamed colon were immediately washed in ice-cold PBS containing 5 mM glucose, pH 7.4. They were then transferred to scintillation vials containing 2 ml of PBS with glucose, pH 7.4, preincubated at 37°C for 3 min, and counted in a scintillation counter after

adding luminol at a concentration of 300 μM. Chemiluminescence was also measured after the addition of PLD (10 μg), PA (10 μM), DG (10 μM), or glycerol (100, 200, 400, 800 mM) to the preincubation buffer. Chemiluminescence was expressed as kilocounts/min/mg protein.

Preparation of polymorphonuclear leukocytes suspension.

Rat PMNs suspension were prepared as described.¹² Briefly, 2 % (w/v) casein sodium (20–25 ml) was injected intraperitoneally in a male Wistar rat (200–250 g) that had been fasted for 24 hours. After 16–18h, the animal was anesthetized with ethyl ether and killed by cardiopuncture. The peritoneum was washed with 50 ml of ice-cold PBS and then washing fluid was collected. The fluid was centrifuged at 150×g for 5 min at 4°C. The pellet was resuspended in 20 ml of ice-cold PBS. The PMNs suspension (8×10⁴ cells/ml) with more than 90% purity was kept on ice until use.

Chemiluminescence of PMNs.

The 500 μl of PMNs suspension was then transferred to scintillation vials containing 1.5 ml of PBS with glucose, pH 7.4, preincubated at 37°C for 3 min, and counted in a scintillation counter after adding luminol at a concentration of 300 μM. Chemiluminescence was also measured after the addition of PLD (10 μg), PA (10 μM), DG (10 μM), or propranolol^{13,14} (300 μM) to the preincubation buffer.

Determination of PA production by PLD.

The assay was carried out essentially as described previously.¹⁵ Briefly, the assay mixture for cabbage PLD contained 0.4 mM C₆-NBD-PC, 50 mM acetic acid, pH 6.0, 10 mM CaCl₂, and 10 μg of enzyme protein in a total volume of 100 μl. The assay mixture for oleate-dependent PLD contained 0.1 mM C₆-NBD-PC, 50 mM sodium dimethylglutaric acid, pH 6.5, 2 mM MgCl₂, 1 mM CaCl₂, and 10 μg of mucosal protein in a total volume of 100 μl. All components except for the enzyme were mixed and sonicated for 10 min. An enzyme protein was added to initiate the reaction, which was allowed to react for 30 min at 30°C before being terminated by adding 3 ml of chloroform/methanol (2 : 1, v/v) and 0.5 ml saline. The mixture was then shaken and centrifuged at 600×g for 10 min. The organic phase was concentrated in a Vapor Mix evaporator (Tokyo Rika, Tokyo, Japan). The lipids were separated by thin-layer chromatography on a Silica Gel 60 plate (Merck, Darmstadt, Germany) with the upper phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (65 : 10 : 15 : 50, v/v).

Preparation of PLD.

As an enzyme source of colonic mucosal PLD, mucosal scrapings were homogenized with Diox 600 homogenizer (Heidolph, Germany) in 5 volumes of 0.25 M sucrose containing 5 mM HEPES, pH 7.2, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride, and then centrifuged at $12,000\times g$ for 20 min. The supernatant was further centrifuged at $100,000\times g$ for 60 min. The resultant pellet was suspended in a minimal volume of the homogenizing buffer and kept at -80°C until use.

Cabbage PLD was prepared from cabbage white leaves according to the method described by Davidson.¹⁶ Briefly, the light-green leaves of cabbages were homogenized with water for 5 min in a blender. The homogenates were squeezed through cheese cloth and centrifuged at $13,000\times g$ for 30 min. The supernatant was then held at 55°C for 5 min, rapidly cooled to 0°C , and centrifuged at $13,000\times g$ for 20 min. The pellet was discarded and the supernatant was diluted with 2 volumes of acetone at -20°C . After 10 min the precipitate was centrifuged down, dissolved in water and cleared of insoluble materials by centrifugation at $13,000\times g$ for 20 min. The supernatant was used as cabbage PLD.

The protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA).

Statistical analysis.

Data were presented as the mean $\pm$ SEM. Statistical analyses were performed according to the Mann-Whitney U-test for comparisons of the data of 2 groups and Bonferroni Correction was performed after Non-repeated Measures ANOVA for comparisons of the data of over 3 groups. Results were considered significant if a P value was less than 0.05; all tests were two-tailed. All statistical analyses were performed on a personal computer with the statistical software ystat 2000. xls for Windows.

Results

Mucosal PLD activity.

Oleate-dependent PLD activity was detected in the colonic mucosa as the phosphatidic acid production and activated in the colitic mucosa (Fig. 1).

Chemiluminescence of colonic mucosa.

Chemiluminescence was measured as a marker of ROMs. All rats treated with DSS, not control rats, showed signs of systemic illness including soft stool and diarrhea. Colons in DSS-treated rats showed mucosal redness and erosions. Luminol-amplified chemiluminescence was significantly higher in the

Fig. 1. Phospholipase D activity in rat colon. Rats were allowed free access to 5% dextran sulfate sodium solution (DSS group) or tap water (control group) as drinking water for 7 days. Colons were excised after the treatment. Mucosal PLD activity was determined as described in Methods. Lane 1: rat brain, lane 2: control mucosa, lane 3: colitic mucosa, lane 4: no protein. PLD: phospholipase D, PA: phosphatidic acid, PC: phosphatidylcholine.

Fig. 2. Chemiluminescence of colitic mucosa in rats. Rats were allowed free access to 5% dextran sulfate sodium solution (DSS group) or tap water (control group) as drinking water for 7 days. Colons were excised after the treatment. Luminol-amplified chemiluminescence of the mucosa was measured as described in Methods. Results are mean $\pm$ SEM of 6 specimens of rat colon in each group. ** $p < 0.01$ compared to the control.

colitic mucosa than in the control mucosa (Fig. 2).

Mucosal MPO activity.

MPO was measured as a marker of neutrophil infiltration. MPO activity in the colonic mucosa was significantly higher in the treated group than in the control group (Fig. 3).

Fig. 3. Myeloperoxidase activity in rat colon. Rats were allowed free access to 5% dextran sulfate sodium solution (DSS group) or tap water (control group) as drinking water for 7 days. Colons were excised after the treatment. Mucosal myeloperoxidase activity was determined as described in Methods. Results are mean $\pm$ SEM of 6 specimens of rat colon in each group. ** $p < 0.01$ compared to the control.

Effects of PLD, PA or DG on chemiluminescence of colonic mucosa.

After the addition of PLD, PA or DG, chemiluminescence of the colitic mucosa was significantly increased (Fig. 4). In contrast, the chemiluminescence

Fig. 4. Effects of phospholipase D, phosphatidic acid and diacylglycerol on mucosal chemiluminescence. Luminol-amplified chemiluminescence of the colitic mucosa was measured in the presence and in the absence of cabbage PLD (10 μ g), PA (10 μ M), or DG (10 μ M). PLD: phospholipase D, PA: phosphatidic acid, DG: diacylglycerol. Results are mean $\pm$ SEM of 6 specimens of rat colon in each group. ** $p < 0.01$ compared to no addition.

of the normal mucosa was not increased even after the addition of PLD, PA or DG (data not shown).

Effects of glycerol on PA production and chemiluminescence.

The addition of glycerol to the PLD assay mixture produced phosphatidylglycerol at the expense of PA production (Fig. 5). In proportion to the inhibition of PA production, glycerol showed a significant dose-dependent inhibition of the chemiluminescence of the colitic mucosa (Fig. 6).

Fig. 5. Effects of glycerol on phosphatidic acid production by phospholipase D. PA production by cabbage PLD was measured in the presence and in the absence (lane 2) of glycerol at the concentration of 100 mM (lane 3), 200 mM (lane 4), 400 mM (lane 5), and 800 mM (lane 6) as described in Methods. Unidentified spots (UI) adjacent to PA were seen in lane 5 and 6. Lane 1: no protein. PLD: phospholipase D, PA: phosphatidic acid, PG: phosphatidylglycerol, PC: phosphatidylcholine.

Effects of PLD, PA or DG on chemiluminescence of PMNs.

Rat PMNs suspension was prepared to directly demonstrate the role of PLD in ROMs production in neutrophils. The chemiluminescence of the PMNs suspension was significantly enhanced after the addition of PLD, PA or DG (Fig. 7).

Effects of propranolol on chemiluminescence of PMNs.

To demonstrate which of the metabolic products by PLD is responsible for chemiluminescence, propranolol, which is known to be a PA phosphohy-

Fig. 6. Effects of glycerol on mucosal chemiluminescence. Mucosal luminol-amplified chemiluminescence in the colitic mucosa was measured in the presence and in the absence of glycerol at the concentration of 100, 200, 400, and 800 mM as described in Methods. Results are mean $\pm$ SEM of 6 specimens of rat colon in each group. * $p < 0.05$, ** $p < 0.01$ compared to no glycerol.

Fig. 7. Effects of phospholipase D, phosphatidic acid and diacylglycerol on chemiluminescence of polymorphonuclear leukocytes. Luminol-amplified chemiluminescence of polymorphonuclear leukocytes suspension was measured in the presence and in the absence of cabbage PLD (10 μ g), PA (10 μ M), or DG (10 μ M). PLD: phospholipase D, PA: phosphatidic acid, DG: diacylglycerol. Results are mean $\pm$ SEM of 6 specimens in each group. * $p < 0.05$, ** $p < 0.01$ compared to no addition.

drolase inhibitor, was added to the assay mixture. The chemiluminescences of PMNs suspension induced by both PLD products, PA and DG, were found to be significantly inhibited by propranolol (Fig. 8).

Discussion

The results of this study suggest that PLD is relevant to ROMs production in mucosal neutrophils in experimental rat colitis. The evidence includes the

Fig. 8. Effects of propranolol on chemiluminescence of polymorphonuclear leukocytes. Luminol-amplified chemiluminescence of polymorphonuclear leukocytes suspension in the presence of PA (10 μ M) or DG (10 μ M) was measured with or without 300 μ M propranolol. PA: phosphatidic acid, DG: diacylglycerol, PPN: propranolol. ** $p < 0.01$ compared to no propranolol.

following: 1) the colitic mucosa had higher chemiluminescence, PLD activity, and MPO activity than normal mucosa; 2) the addition of exogenous PLD, PA, or DG, enhanced the colitic, but not the normal colonic, chemiluminescence; 3) the chemiluminescence of the colitic mucosa was inhibited by glycerol which prevents PA formation by PLD; 4) the chemiluminescence of PMNs suspension was enhanced by the addition of exogenous PLD, PA, or DG; 5) the PA and DG induced enhancements were inhibited by propranolol.

In humans an important hallmark of inflammatory bowel disease is mucosal neutrophil infiltration from which ROMs are released to damage colonic mucosa.^{4,5,17} This may apply to the rat colitis model.^{18,19} This model shows the enhanced chemiluminescence and MPO activity of the colitic mucosa, indicating that neutrophils infiltrating into the mucosa produced ROMs and induced colitis. The previously mentioned evidence 1), 2), 3), indicates that PLD and its products are relevant to ROMs production in colitic mucosa. Furthermore, the evidence 4), 5), suggests that the PLD in neutrophils plays a role in ROMs production. In this context, this present study may provide a new target, PLD in neutrophils, for inhibiting ROMs production and consequent mucosal damage in the colitis of both rats and humans. Moreover, propranolol or its derivatives may become a new candidate agent for the treatment of colitis.

Results in this study are apparently inconsistent with previous results⁸ that showed mucosal PLD activity is not proportional to mucosal MPO activity and

that PLD in phagocytic cells rather than in neutrophils may be more important to mucosal injury in experimental rat colitis.⁸ This inconsistency may be explained by the difference in the type of experimental colitis used in these studies. The former study used a model of acetic acid-induced colitis because of its simplicity and reproducibility,⁸ but this model exhibits acute type colitis with a relatively specific reaction to organic acid.⁹ This reaction may have emphasized the action of PLD in phagocytic cells rather than in neutrophils.⁸ In the present study a model of DSS-induced colitis was selected instead because this model induced by an immunological epithelial injury has no such specific reaction and exhibits the hallmark of chronic colitis, infiltration of neutrophils in the mucosal and submucosal layer, and is more similar to inflammatory bowel disease in humans.⁹

The previous study demonstrated that oleate-dependent PLD is activated and that its activity correlates to colonic damage in acetic acid-induced colitis in rats.⁸ The present study also found the activation of this type of PLD isoform in DSS-induced colitis, but immunological trials (data not shown) could not specify PLD isoform that is most relevant to colitis among the three types of PLD: PLD1, PLD2, and oleate-dependent PLD.^{8,20-23} This may be due to the scarcity of PLD enzymes for detection and this specification remains to be determined in the near future.

Preliminary data found that propranolol inhibited the chemiluminescence of PMNs themselves without exogenous PA and DG by 24% (data not shown). Because PMNs prepared from ascites are considered to be already activated and produced ROMs, propranolol may have exhibited this inhibition by eliminating the effects of PA and DG produced via PLD in the cells. Even after deducing this inhibition, the results in figure 7 and 8 still suggest that propranolol inhibited chemiluminescence enhanced by both PA and DG added exogenously. Because PA derived from phosphatidylcholine by PLD is reported to activate NADPH-oxidase and produces superoxide,^{24,25} these results cannot be explained by the well-known action of propranolol as an inhibitor of the PA phosphohydrolase catalyzing conversion of PA to DG. Propranolol has recently been reported to be an inhibitor of protein kinase C²⁶ which is implicated in NADPH-oxidase activation;^{12,27-30} hence protein kinase C inhibition by propranolol in the downstream of PA may explain the result of the current study. This explanation may also apply to the case of DG,^{12,31,32} because this lipid is known to activate protein kinase C. Inhibition of protein kinase C by propranolol may eliminate the enhancement of chemiluminescence caused by DG. An alternative explanation is that

propranolol may reduce ROMs production as a consequence of the membrane-stabilization of neutrophils.³³ However, the existence of other unidentified targets of propranolol in ROMs production can now no longer be denied.

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